Adenosine, Via A2B Receptors, Inhibits Human (P-SMC) Progenitor Smooth Muscle Cell Growth

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Abstract—c-Kit+ progenitor smooth muscle cells (P-SMCs) can develop into SMCs that contribute to injury-induced neointimal thickening. Here, we investigated whether adenosine reduces P-SMC migration and proliferation and whether this contributes to adenosine's inhibitory actions on neointima formation. In human P-SMCs, 2-chloroadenosine (stable adenosine analogue) and BAY60-6583 (A_{2B} agonist) inhibited P-SMC proliferation and migration. Likewise, increasing endogenous adenosine by blocking adenosine metabolism with erythro-9-(2-hydroxy-3-nonyl) adenine (inhibits adenosine deaminase) and 5-iodotubercidin (inhibits adenosine kinase) attenuated P-SMC proliferation and migration. Neither N^6-cyclopentyladenosine (A_1 agonist), CGS21680 (A_{2A} agonist), nor N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (A_3 agonist) affected P-SMC proliferation or migration. 2-Chloroadenosine increased cyclic AMP, reduced Akt phosphorylation (activates cyclin D expression), and reduced levels of cyclin D1 (promotes cell-cycle progression). Moreover, 2-chloroadenosine inhibited expression of Skp2 (promotes proteolysis of $p27^{Kip1}$) and upregulated levels of $p27^{Kip1}$ (negative cell-cycle regulator). A_{2B} receptor knockdown prevented the effects of 2-chloroadenosine on cyclic AMP production and P-SMC proliferation and migration. Likewise, inhibition of adenylyl cyclase and protein kinase A rescued P-SMCs from the inhibitory effects of 2-chloroadenosine. The inhibitory effects of adenosine were similar in male and female P-SMCs. In vivo, peri-arterial (rat carotid artery) 2-chloroadenosine (20 μmol/L for 7 days) reduced neointimal hyperplasia by 64.5% ($P<0.05$; intima/media ratio: control, 1.4±0.02; treated, 0.53±0.012) and reduced neointimal c-Kit+ cells. Adenosine inhibits P-SMC migration and proliferation via the A_{2B} receptor/cyclic AMP/protein kinase A axis, which reduces cyclin D1 expression and activity via inhibiting Akt phosphorylation and Skp2 expression and upregulating $p27^{kip1}$ levels. Adenosine attenuates neointima formation in part by inhibiting infiltration and proliferation of c-Kit+ P-SMCs. (***Hypertension*. 2020;75:109-118. DOI: 10.1161/HYPERTENSIONAHA.119.13698.)** • **Online Data Supplement**

Key Words: adenosine ■ adult stem cells ■ neointima ■ phosphorylation ■ vascular remodeling

Neointimal hyperplasia is a vascular remodeling process involving multiple cell types that is particularly aggressive in hypertension and contributes to atherosclerotic coronary artery disease.[1] Although dysfunction of endothelial cells and abnormal growth of vascular smooth muscle cells (SMCs) contributes to the cause of neointimal formation,[1] c-Kit+ progenitor cells originating from both vascular and extravascular (bone marrow) sources may also contribute to the pathophysiology of injury-induced neointimal hyperplasia.[2–5] In this context, CD34+/c-Kit+ mononuclear cells can differentiate (in response to growth factors like platelet derived growth factor, which is released during injury) into cells that express a SMC phenotype and subsequently contribute to neointimal formation by migrating to the site of injury and proliferating.[6–10] While the relative contribution of progenitor SMCs (P-SMCs) in neointimal formation remains an open question, studies conducted in the last decade suggest that P-SMCs derived from CD34+/c-Kit+ mononuclear cells contribute to neointimal thickening by 6% to 60%.[4,5] Although circulating bone marrow/hematopoietic-derived CD34+/c-Kit+ cells are postulated to be the key extravascular source of neointimal P-SMCs,[2,5] local c-Kit+ cells of vascular adventitial origin may also contribute to neointimal formation.[2]

Importantly, dysfunction and abnormal growth of vascular cells involved in neointimal hyperplasia is attenuated by endogenous molecules generated by vascular injury.[1] In this regard, adenosine, an endogenous purine nucleoside also known as a retaliatory metabolite, is a regulator of cell function that is generated in response to cell injury and stress and may provide vascular protection.[11,12] The biological effects of adenosine are mediated via multiple adenosine receptors identified

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From the Department of Obstetrics and Gynecology, Clinic for Reproductive Endocrinology, University Hospital Zurich (R.K.D., I.B., R.S., B.L., B.I., M.R.); Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland (R.K.D.); Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine (R.K.D., D.G.G., Z.M., E.K.J.); and NMI Naturwissenschaftliches und Medizinisches Institut an der Universität Tübingen, Reutlingen, Germany (J.F.).

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Correspondence to Raghvendra K. Dubey, Department for Reproductive Endocrinology, University Hospital Zurich, Areal-Wagi Schlieren, Wagistrasse 14, 8952 Schlieren, Switzerland. Email raghvendra.dubey@usz.ch

as A_1, A_{2A}, A_{2B}, and A_3.[11,12] Work by us and others show that adenosine, via A_{2B} receptors, inhibits SMC growth and balloon injury–induced neointimal formation in rats.[13,14]

A_{2B} receptors are referred to as the low affinity adenosine receptor because the binding affinity of A_{2B} receptors for adenosine is lower than that of A_1, A_{2A}, and A_3 receptors. Nonetheless, the functional relevance of A_{2B} receptors is supported by its link to key intracellular signaling pathways.[15] Moreover, A_{2B} receptors are highly responsive to biological processes and mediators, which induce its expression by both transcriptional and post-transcription mechanisms.[15] For example, during hypoxia and inflammation, there is increased production of adenosine and augmented expression of A_{2B} receptors, in part because adenosine increases A_{2B} receptor expression. These responses suggest that A_{2B} receptors may play a dynamic pathophysiological role in many diseases.[15]

There is mounting evidence that the adenosine/A_{2B} receptor system plays a particularly important role in vascular diseases. For example, A_{2B}-receptor expression is increased following vascular injury,[14] and knockdown of A_{2B} receptors as well as CD73 (an ecto-nucleotidase that converts 5'-AMP to adenosine on cell surfaces) results in vessel wall thickening.[16–18] Also, treatment with A_{2B}-receptor agonists prevents injury-induced neointima formation,[14] and CD73-derived adenosine prevents cardiac fibrosis and heart failure.[18] As reviewed before,[13] via A_{2B} receptors, adenosine inhibits abnormal growth of multiple cell types involved in cardiovascular and renal diseases. Specifically, activation of A_{2B} receptors inhibits the growth of human glomerular mesangial cells (phenotypically similar to vascular SMCs), rat preglomerular SMCs, and rat cardiac fibroblasts. In addition, and again via A_{2B} receptors, adenosine actually stimulates the proliferation of cells that promote vascular health. In this regard, studies show that the adenosine/A_{2B}-receptor system augments growth of rat aortic,[13] rat renal microvascular,[19] and porcine coronary endothelial cells,[13] as well as human renal epithelial cells.[18] Additionally, adenosine has several other desirable tissue-protecting actions such as promoting neovascularization[20] and preventing and reducing inflammation and hypoxia.[15] Hence, adenosine per se, adenosine receptor agonists, or adenosine-modulating drugs (ie, the broad class of adenosinergic drugs) may be useful for preventing and treating a number of cardiovascular and renal diseases induced by hypertension, particularly those associated with excessive proliferation or migration of neointimal cells (including SMCs or progenitor SMCs) or immune cells.

As P-SMCs derived from CD34+/c-Kit+ mononuclear cells contribute to neointimal formation,[4,5] it is conceivable that the ability of adenosine to attenuate neointimal hyperplasia is mediated in part by adenosine's effects on P-SMC proliferation and migration. In support of this hypothesis, adenosine, via A_{2B} receptors, reduces vascular adhesion of inflammatory cells,[21]induces growth of CD34+ derived progenitor endothelial cells, and promotes angiogenesis[20–22]; however, whether adenosine influences growth of CD34+/c-Kit+ derived P-SMCs remains unknown. Moreover, the impact of adenosine on the presence of c-Kit+ cells in injury-induced neointimal formation is unknown. As via A_{2B} receptors adenosine inhibits SMC growth and induces growth of endothelial and progenitor endothelial cells,[13,14,20,22] and A_{2B} receptor expression is increased following vascular injury,[14] we hypothesize that inhibitory actions of adenosine on CD34+/c-Kit+ P-SMCs contributes to adenosine's overall inhibitory effects on neointimal formation.

Adenosine inhibits growth of vascular SMCs by downregulating mitogen activated Akt-phosphorylation and the subsequent activation of cyclin D1 by inhibiting S-phase kinase associated protein-2 (Skp2; promotes the polyubiquitination of $p27^{Kip1}$ and accelerates $p27^{Kip1}$ degradation)[13] and upregulating/increasing $p27^{kip1}$, a negative regulator of cell cycle progression. Additionally, similar to Wu et al,[23] our findings demonstrate that the antimitogenic cascade of events driven by adenosine in SMCs is triggered by A_{2B}-activated release of cyclic AMP (cAMP) and involves PKA (protein kinase A).[13] In this context, cAMP downregulates the expression of Skp2,[23] and thereby increases the levels of $p27^{Kip1}$. In addition, PKA can interfere with signaling cascades that phosphorylate (activate) Akt,[24] thus providing additional mechanisms for inhibiting cyclin D signaling. We hypothesize that similar to SMCs, adenosine inhibits growth of CD34+/c-Kit+ derived P-SMCs by engaging the A_{2B} receptor/adenylyl cyclase/cAMP/PKA pathway.

The goals of the present study were to investigate: (1) whether adenosine inhibits proliferation and migration of P-SMCs derived from circulating human CD34+/c-Kit+ mononuclear cells; (2) the underlying receptors and mechanism(s) involved; and (3) whether adenosine inhibits the number of c-Kit+ cells within the neointima after balloon injury in rats.

Methods

The authors declare that all supporting data are available within the article (online-only Data Supplement).

Details of all methods used are available in the online-only Data Supplement. It provides information on materials and reagents used (Table S1 in the online-only Data Supplement), culture of human P-SMCs from peripheral CD34+ mononuclear cells, cell growth (^{3}H-thymidine incorporation, ^{3}H-proline incorporation, and cell number) assays, cell migration, Western blotting for intracellular proteins, siRNA mediated A_{2B}-receptor knockdown, cAMP assay, immunomagnetic isolation of c-Kit+ cells; carotid artery injury studies, and flow cytometry.

Culture of P-SMCs

P-SMCs were cultured from CD34+ mononuclear cells magnetically isolated as previously described.[25–29] Briefly, peripheral blood (10 mL) was collected in heparinized tubes from healthy women (n=13; mean age 36±5, nonsmokers). Blood collection to isolate and culture progenitor cells was approved by the institutional ethics commission at University Hospital Zurich (stv-Nr 04/2006) and Kantonal etic-commission (KEK) with written consent obtained before blood collection. Similarly, the role of adenosine and A_{2B} receptors in regulating P-SMC growth was also assessed in P-SMCs cultured from peripheral CD34+ cells from healthy male donors (n=3, mean age 27.3±5.4).

Carotid Artery Injury Studies

Balloon catheter-induced carotid artery injury in rats was performed as described previously by us[13] and conformed to internationally accepted standards and guidelines set by the animal welfare committee and approved by the institutional review board. To assess the impact of Cl-Ad on P-SMC infiltration after balloon injury, animals (placebo n=7, treated n=7) receiving the vehicle or Cl-Ad were sacrificed and perfused-fixed 7 days after balloon injury and sections immunostained for c-Kit+ cells to assess their contribution to neo-intimal thickening. The sections of the stained carotid arteries were viewed and analyzed in a blinded fashion.

Statistics

Treatment effects on cross-sectional areas were analyzed by using ANOVA or the nonparametric Kruskal-Wallis test. Expression and growth data was analyzed using ANOVA and statistical significance ($P<0.05$) was calculated using Fisher Least Significant Difference test. All growth experiments were performed in triplicates or quadruplicates with 3 to 4 separate cultures.

Results

The experiments described below required the use of a large number of adenosinergic drugs. To improve readability, here we summarize the activity of these pharmacological probes and provide abbreviations: 2-Chloroadenosine (Cl-Ad) is a stable adenosine analogue and nonselective adenosine receptor agonist; 5'-N-ethylcarboxamidoadenosine is a nonselective adenosine receptor agonist; 5'-N-methylcarboxamidoadenosine (MECA) is a nonselective adenosine receptor agonist; N^6-cyclopentyladenosine (CPA) is a selective A_1 receptor agonist; CGS21680 (CGS) is a selective A_{2A} receptor agonist; BAY60-6583 (BAY) is a selective A_{2B} receptor agonist; 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA) is a selective A_3 receptor agonist; 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) is a selective A_1 receptor antagonist; SCH442416 (SCH) is a selective A_{2A} receptor antagonist; MRS1754 (MRS) is a selective A_{2B} receptor antagonist; VUF5574 (VUF) is a selective A_3 receptor antagonist; Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) increases endogenous adenosine by inhibiting adenosine deaminase and thus reducing the metabolism of adenosine to inosine; 5-iodotubercidin (IDO) increases endogenous adenosine by inhibiting adenosine kinase and thus reducing the metabolism of adenosine to 5'-AMP.

As shown in Figure 1A, progenitor cells magnetically isolated from mononuclear cells using a CD34 separation kit expressed CD34 (green) and c-Kit (orange). As shown in Figure S1, soon after plating, CD34+/c-Kit+ cells developed a hill-and-valley appearance. Colonies of cells with elongated smooth muscle like morphology were selectively isolated, characterized, and cultured. P-SMCs had characteristic elongated and spindle shaped morphologically similar to SMCs, but not progenitor endothelial cells (Figure S1). After the third passage, P-SMCs took on an even more SMC-like morphology (elongated and spindle shaped; Figure S1). P-SMCs in third passage were further immune-characterized for SMC phenotype. As shown in Figure 1B through 1D, P-SMCs showed positive immunostaining for alpha smooth muscle actin, vimentin, and desmin and were negative for von Willibrand factor, an endothelial cell marker. Importantly, Western blotting of P-SMCs lysates revealed expression of PDGFR-B and SM22-α (Figure 1E), established markers for P-SMCs.[4,5]

The expression profile of adenosine receptor subtypes determines the overall pharmacology and cellular actions of adenosine. Hence, we assessed their presence in human P-SMCs. As shown in Figure 1F, Western blotting detected strong bands for A_1 and A_{2B} receptors, a faint band for A_{2A} receptors and no signal for A_3 receptors. These findings suggest a potential role for A_{2B} receptors in mediating adenosine actions in P-SMCs.

Treatment of P-SMCs with Cl-Ad (0.01–1 μmol/L; stable adenosine analogue) for 4 days concentration-dependently attenuated cell proliferation (Figure 2A). Using various

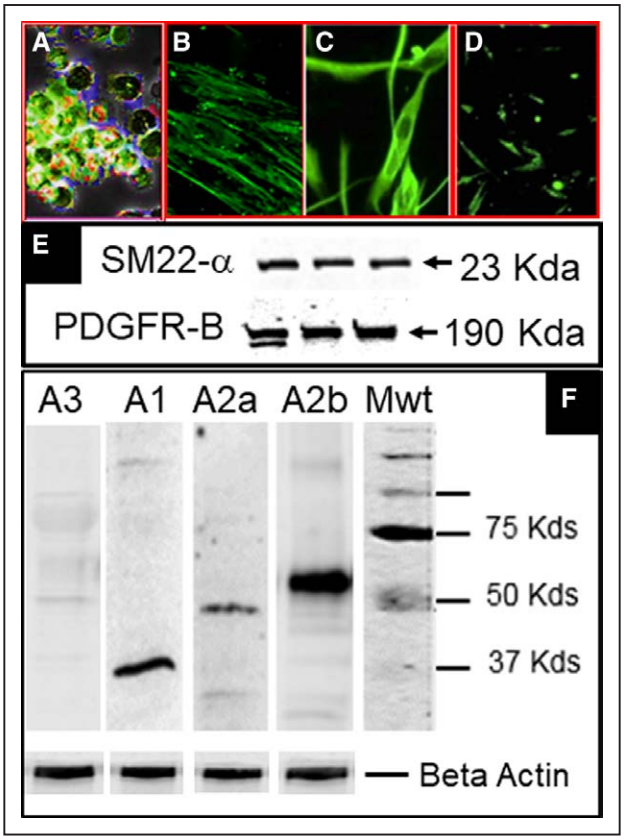

Figure 1. A, Depicts expression of CD34 (green) and c-Kit (orange) in magnetically separated CD34+ mononuclear cells obtained from human blood. Soon after plating, CD34+/c-Kit+ cells developed a hill-and-valley appearance. Colonies of cells with elongated smooth muscle–like morphology were selectively isolated, characterized, and cultured. Progenitor smooth muscle cells (P-SMCs) had characteristic elongated and spindle shaped morphologically similar to SMCs, but not progenitor endothelial cells. After the third passage, P-SMCs took on an even more SMC-like morphology (elongated and spindle shaped; Figure S1). P-SMCs in third passage showed positive immunostaining for alpha smooth muscle actin (**B**), vimentin (**C**), and desmin (**D**) and were negative for von Willibrand factor, an endothelial cell marker. Western blotting of P-SMC lysates revealed expression of platelet derived growth factor receptor-B and SM22-α (**E**), established markers for P-SMCs. Western blotting detected strong bands for A_1 and A_{2B} receptors, a faint band for A_{2A} receptors and no signal for A_3 receptors (**F**). Molecular weight numbers in blots are in kilodaltons (Kda or Kds).

pharmacological agents (adenosine receptor subtype selective and nonselective agonists and antagonists), we further assessed the role of all adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) in mediating the antimitogenic effects of adenosine in P-SMCs. The highest (1 μmol/L) concentrations of CPA (A_1-receptor selective agonist), CGS (A_{2A}-receptor selective agonist), and IB-MECA (A_3-receptor selective agonist) failed to inhibit cell proliferation (Figure 2A). MECA was more potent than 5'-N-ethylcarboxamidoadenosine (both are nonselective adenosine receptor agonists; Figure 2A). MRS (A_{2B}-receptor selective antagonist), but not DPCPX (A_1-receptor selective antagonist), SCH (A_{2A}-receptor selective antagonist) or VUF (A_3-receptor selective antagonist), blocked the inhibitor effects of Cl-Ad on ^{3}H-thymidine incorporation, an index of DNA synthesis (all antagonists at 100 nmol/L; Figure 2B). Moreover, the inhibitory effects of Cl-Ad on cell number and ^{3}H-proline

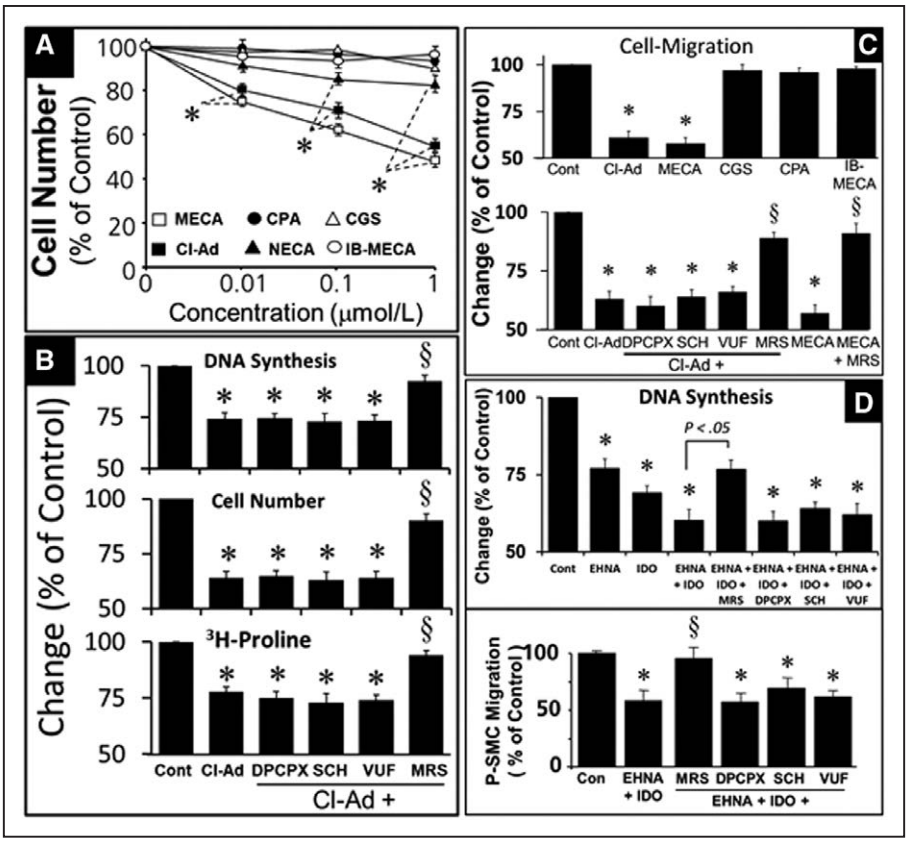

Figure 2. Left: (**A**) depicts concentration-response relationships for the inhibition of cell number by Cl-Ad, N-methylcarboxamidoadenosine (MECA), IB-MECA, N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), and CGS21680 in progenitor smooth muscle cells (P-SMCs). **B**, Depicts the effects of Cl-Ad (1 μmol/L) on ^{3}H-thymidine incorporation (top; DNA synthesis), cell number (middle), and ^{3}H-proline incorporation (panel; collagen production) in the presence and absence of DPCPX, SCH442416, VUF5574 , and MRS1754 (100 nmol/L) in P-SMCs. *P<0.05 vs no treatment; §Significant reversal of inhibitory effects. Values represent mean±SEM from 3 to 4 separate experiments, each conducted in triplicate. Right: upper of (**C**) depicts the effects of Cl-Ad, MECA, CPA, CGS, and IB-MECA (1 μmol/L) on human P-SMC migration. The inhibitory effects of Cl-Ad were mimicked by MECA, but not by CPA, CGS, or IB-MECA. Lower of (**C**) shows that the inhibitory effects of Cl-Ad on P-SMC migration were reversed by MRS, but not by DPCPX, SCH, or VUF. Also, the effects of MECA on migration of human P-SMCs were blocked by MRS. Upper (**D**) show that EHNA (5 μmol/L), IDO (0.1 μmol/L), and EHNA plus IDO inhibited DNA synthesis (^{3}H-thymidine incorporation) by human P-SMCs and lower (**D**) shows that EHNA plus IDO inhibited migration. The inhibitory effects of EHNA and IDO on DNA synthesis were significantly enhanced when the adenosine catabolism inhibitors EHNA and IDO were combined. Moreover, the effects of EHNA+IDO were reversed by MRS, but not by SCH, DPCPX or VUF, suggesting that endogenous adenosine inhibits P-SMC growth and migration via A_{2B} receptors. *P<0.05 vs control; §Significant reversal of the inhibitory effects. Values represent mean±SEM from 3 to 4 separate experiments, each conducted in triplicate.

incorporation (index of collagen synthesis) were reversed by MRS, but not by DPCPX, SCH, or VUF (Figure 2B).

Treatment with Cl-Ad and MECA (nonselective adenosine receptor agonist), but not CPA (selective A_1 agonist), CGS (selective A_{2A} agonist), or IB-MECA (selective A_3 agonist), inhibited P-SMC migration (Figure 2C, top) and the inhibitory effects of Cl-Ad and MECA on cell migration were blocked by MRS (Figure 2C, bottom). Neither DPCPX, SCH, nor VUF affected the inhibitory actions of Cl-Ad on P-SMC migration (Figure 2C, bottom).

To assess whether endogenous adenosine has inhibitory effects on P-SMCs, cells were treated with EHNA (blocks adenosine deaminase), IDO (blocks adenosine kinase) or both to increase endogenous adenosine. Treatment with EHNA as well as IDO inhibited P-SMC mitogenesis and these effects were enhanced in cells treated with EHNA plus IDO (Figure 2D, upper). The antimitogenic effects of EHNA plus IDO on P-SMC were abrogated by MRS, but not by DPCPX, SCH or VUF (Figure 2D, upper). The inhibitory effects of Cl-Ad on P-SMC migration were mimicked by endogenous adenosine induced by treatment with EHNA plus IDO in P-SMCs (Figure 2D, lower). The inhibitory actions of EHNA plus IDO on P-SMC migration were abrogated by the A_{2B} antagonist MRS, but not by DPCPX, SCH or VUF (Figure 2D, lower). Trypan blue exclusion tests demonstrated that none of the aforementioned treatments altered cell viability. These findings indicate that in human P-SMCs, A_{2B} receptors dominate the pharmacology of adenosine leading to inhibition of cell proliferation, DNA synthesis, collagen synthesis, and cell migration.

We have previously shown that active crosstalk between Akt-phosphorylation, Skp2, $p27^{Kip1}$, and cyclin D1 plays a key role in mediating the growth inhibitory effects of adenosine in human coronary artery SMCs.[13] In the present study (Figure 3), treatment of human P-SMCs with Cl-Ad, as well as MECA, inhibited Akt phosphorylation and decreased expression of Skp2 (F-box protein of SCF-Skp2 ubiquitin ligase responsible for polyubiquitination of and subsequent proteolysis of $p27^{Kip1}$) and upregulated levels of $p27^{Kip1}$ ($p27^{Kip1}$ is a negative regulator of cell cycle progression which blocks cyclin action). This effect was to paralleled by inhibition of cyclin D1 expression (Figure 3). Moreover, the modulatory effects of Cl-Ad and MECA on Akt phosphorylation, Skp2, $p27^{Kip1}$, and

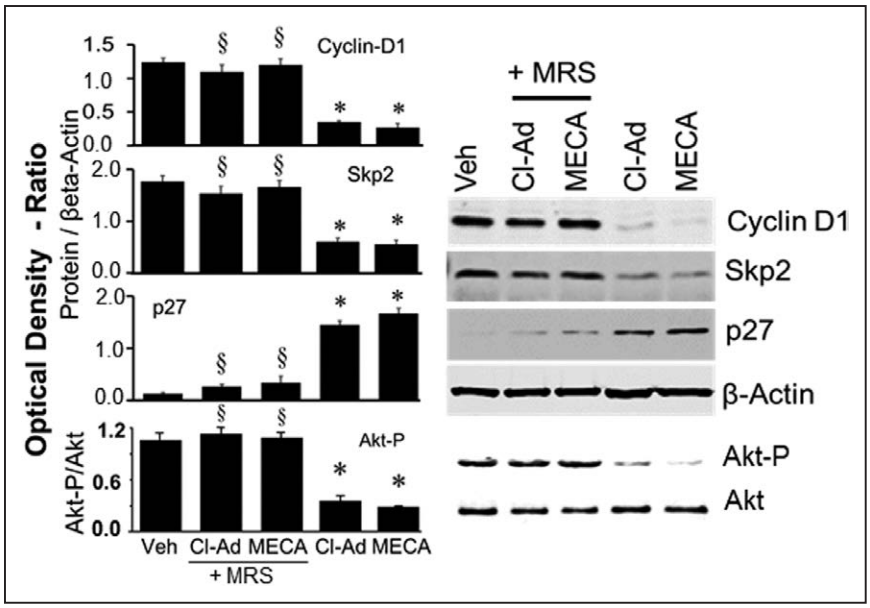

Figure 3. Figure depicts Western blots showing the effects of Cl-Ad (0.5 μmol/L), Cl-Ad plus MRS1754 (100 nmol/L), N-methylcarboxamidoadenosine (MECA; 0.5 μmol/L), and MECA plus MRS1754 on levels of phosphorylated Akt (Akt-P), total Akt, cyclin D1, $p27^{Kip1}$ (p27), and Skp2 expression in human progenitor smooth muscle cells (P-SMCs). Subconfluent monolayers of P-SMCs were growth arrested for 36 h in 0.4% BSA and were treated and stimulated with 2.5% FBS for 48 h. Cell lysates were subsequently analyzed by Western blotting. Bar graphs depict the optical density ratio, normalized to appropriate controls, for the Western blots. *P<0.05 vs control; §Significant reversal of the inhibitory effects. The experiments were repeated 3× with separate cultures.

cyclin D1 were blocked by MRS (Figure 3), implying a role for A_{2B} receptors in mediating the inhibitory effects of adenosine on cell cycle progression in human P-SMCs.

To further assess the role of A_{2B} receptors in P-SMCs, we used siRNA to knockdown the expression of A_{2B} receptors (Figure 4A). At the functional level, Cl-Ad increased cAMP production in P-SMCs (Figure 4B). As stimulation of A_{2B} adenosine receptors are known to induce cAMP in SMCs, we assessed the effects of A_{2B} receptor knockdown with siRNA on Cl-Ad-induced cAMP in P-SMCs. Downregulation of A_{2B} receptors in P-SMCs by siRNA (Figure 4B) blocked Cl-Ad induced cAMP production; whereas, treatment with nonactive control siRNA had no effect (Figure 4B). Downregulation of A_{2B} receptors by siRNA abrogated the inhibitory effects of Cl-Ad, MECA, and EHNA plus IDO, but not 8-bromo-cAMP, on DNA synthesis (Figure 4C). These results further support the conclusion that A_{2B} receptors mediate the antimitogenic effects of adenosine. We further elucidated the role of adenylyl cyclase and protein kinase A in mediating the inhibitory effects of Cl-Ad on P-SMC mitogenesis. Treatment of P-SMCs with Cl-Ad inhibited mitogenesis in the absence (Figure 4D), but not in the presence (Figure 4D), of the adenylyl cyclase inhibitor myristoylated trifluoroacetate or the protein kinase A inhibitor 2',5'-dideoxyadenosine.

To assess whether sex influences the inhibitory actions of adenosine on P-SMC growth, we conducted experiments in P-SMCs cultured from peripheral CD34+ cells obtained from males. P-SMCs from males expressed A_{2B} receptors and treatment with Cl-Ad, MECA and BAY (selective A_{2B} receptor agonist), but not CGS, CPA or IB-MECA, inhibited P-SMC proliferation (Figure 5A). The concentration-dependent inhibitory effects of BAY were of similar magnitude in female and male P-SMCs (Figure 5B) and were blocked by MRS. Both Cl-Ad and BAY inhibited migration of (Figure 5C) and DNA synthesis by (Figure 5D) male P-SMCs. Similar effects were observed in male P-SMCs treated with EHNA+IDO to induce endogenous adenosine (Figure 5C and 5D). Moreover, the inhibitory effects of Cl-Ad, BAY, and EHNA plus IDO on P-SMC migration and DNA synthesis were reversed by MRS. Stimulation of A_{2B} adenosine receptors with Cl-Ad as well as BAY induced cAMP in male P-SMCs and these actions were blocked by MRS (Figure 5D). Moreover, BAY inhibited mitogenesis in the absence (Figure 5D), but not in the presence (Figure 5D), of the adenylyl cyclase inhibitor myristoylated trifluoroacetate or the protein kinase A inhibitor 2',5'-dideoxyadenosine.

Analysis of carotid arteries after morphometric evaluation showed significant intimal thickening after balloon injury, and this was abrogated in rats treated with Cl-Ad for 7 days (Figure 6A). Compared with the placebo group (n=7; intima 35,256±9680 pixels), the neointima formation was reduced by ≈64.5% in rats receiving periarterial Cl-Ad (n=7; 12517±4822 pixels; P<0.05 versus placebo). Also, the intimal/medial ratio was significantly reduced from 1.4±0.02 in placebo group to 0.53±0.55 in animals receiving Cl-Ad (Figure 6A). In carotid arteries obtained from animals receiving placebo, c-Kit+ cells were observed in neo-intimal areas (≈16% of total cells; Figure 6B). As compared with the placebo group, a significant decrease in c-Kit+ cells was observed in arteries obtained from animals treated with Cl-Ad (Figure 6B). Moreover, c-Kit+ cells were also present in the adventitial areas (Figure 6C). To assess whether treatment with Cl-Ad modulates bone marrow–derived c-Kit+ cells, we also performed fluorescence-activated cell sorting analysis in marrow-derived mononuclear cells. As shown in Figure 6D, treatment with Cl-Ad did not significantly modulate the number of CD34+ and c-Kit+ marrow cells. Moreover, adenosine A_{2B} receptors were expressed in lysates of c-Kit+ cells isolated by immune-magnetic separation from rat aortas and bone marrow (Figure 6E). Treatment with Cl-Ad was not associated with any toxic adverse effects, as we have previously shown.[13]

Discussion

Here, we report the novel finding that adenosine, via A_{2B} receptors, attenuates P-SMC proliferation and migration. The importance of this discovery is underscored by the fact that circulating marrow-derived progenitor cells contribute to injury-induced vascular remodeling.[4,5] In particular, CD34+/c-Kit+ progenitor cells acquire a SMC phenotype and thereby contribute to injury-induced neointimal thickening.[2,4,5] Thus,

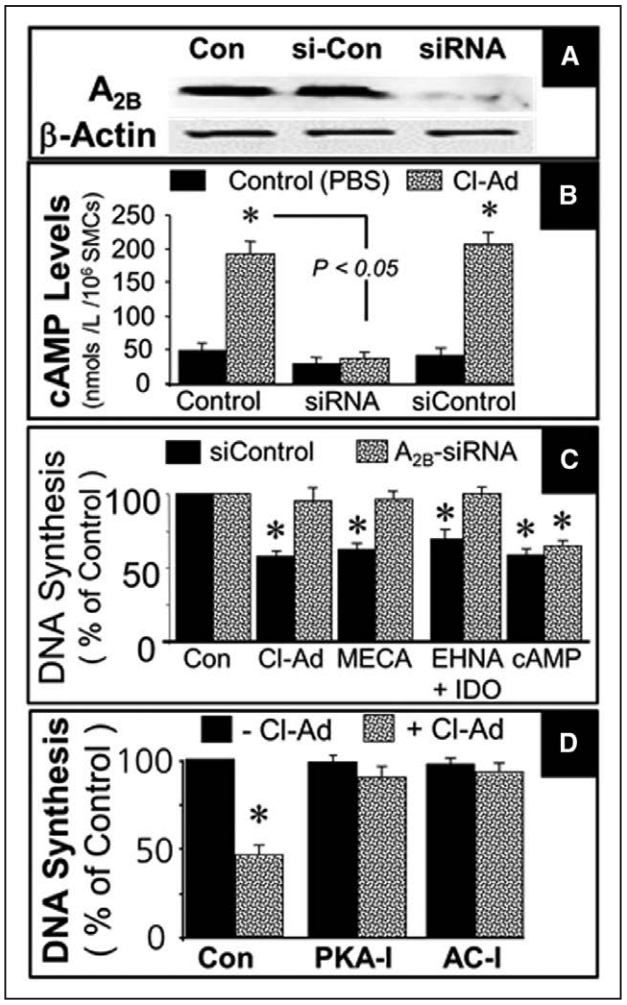

Figure 4. **A**, Shows a Western Blot depicting the downregulation of A_{2B} receptor expression in human progenitor smooth muscle cells (P-SMCs) by siRNA against A_{2B} receptors (no treatment with siRNA [Con]; treated with negative-control siRNA [si-Con]; treated with siRNA against A_{2B} receptor [siRNA]). **B**, Depicts the effects of siRNA against A_{2B} receptors on the stimulatory effects of Cl-Ad (1 μmol/L) on cAMP levels in P-SMCs (no treatment with siRNA [Control]; treated with negative-control siRNA [siControl]; treated with siRNA against A_{2B} receptor [siRNA]). **C**, Depicts inhibitory effects of Cl-Ad (Cl-Ad; 1 μmol/L), MECA (1 μmol/L), 8-bromo-cyclic AMP (cAMP; 10 μmol/L), and Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; 10 μmol/L) plus 5-iodotubercidin (0.1 μmol/L) on DNA synthesis in the absence and presence of A_{2B} receptor siRNA in P-SMCs (no treatment with agonists [Con]; treated with negative-control siRNA [siControl]; treated with siRNA against A_{2B} receptors [A_{2B}-siRNA]). In (**D**), bar graphs depict DNA synthesis in P-SMCs treated (+Cl-Ad) or not (-Cl-Ad) with Cl-Ad (0.5 μmol/L) in the presence or absence of a protein kinase A inhibitor (myristoylated trifluoroacetate, frag 14-22; 10 μmol/L; PKA-I) or adenylyl cyclase inhibitor (2',5'-dideoxyadenosine; 1 μmol/L; AC-I) for 36 h. Both PKA-I and AC-I reversed the inhibitory effects of Cl-Ad on DNA synthesis in P-SMCs. *P<0.05 vs no agonist. Values represent mean±SEM from 3 separate experiments, each conducted in quadruplicates.

agents that inhibit the infiltration and proliferation of CD34+/c-Kit+ derived SMCs in the vasculature may protect against cardiovascular disease.

Our experiments demonstrate that both exogenous and endogenous adenosine inhibited the mitogen-induced activity of P-SMCs derived from human CD34+/c-Kit+ mononuclear cells. In support of this conclusion, we observed that treatment of P-SMCs either with a metabolically stable adenosine analog (Cl-Ad) or with agents that increase endogenous adenosine inhibited P-SMC DNA synthesis, cell proliferation, collagen synthesis, and cell migration. Importantly, Cl-Ad significantly decreased the presence of c-Kit+ cells within the neointima after balloon injury.

Our results also support the conclusion that adenosine inhibits proliferation and migration of P-SMCs via activation of A_{2B} receptors. CPA, CGS, and IB-MECA are selective A_1-receptor, A_{2A}-receptor, and A_3-receptor agonists, respectively; and DPCPX, SCH and VUF are selective A_1-receptor, A_{2A}-receptor, and A_3-receptor antagonists, respectively. As neither CPA, CGS, nor IB-MECA inhibited P-SMC proliferation or migration and neither DPCPX, SCH nor VUF blocked the inhibitory effects of Cl-Ad on P-SMC proliferation or migration, it is highly unlikely that A_1, A_{2A}, or A_3 receptors mediate the antimitogenic or antimigratory effects of adenosine on human P-SMCs. On the contrary, BAY, a specific A_{2B}-receptor agonist, inhibited P-SMC growth. The facts that BAY and MECA mimicked the effects of Cl-Ad on P-SMC proliferation and migration and the observation that MRS, a specific A_{2B} receptor antagonist, attenuated the inhibitory effects of Cl-Ad, BAY, and MECA on P-SMCs corroborate the conclusion that A_{2B} receptors mediate the inhibitory effects of adenosine on P-SMC proliferation. This conclusion is further supported by our findings that the inhibitory effects of MECA and Cl-Ad on P-SMC proliferation were blocked by siRNA against A_{2B} receptors.

Multiple promitogenic pathways, including Akt, promote mitogen-induced proliferative responses at sites of vascular dysfunction, damage, or injury. These early signaling pathways trigger proliferation of cells, including SMCs, by upregulating progrowth cell cycle regulatory proteins, such as cyclin D, and downregulating negative cell cycle regulatory proteins, such as $p27^{Kip1}$, that retard cell-cycle progression.[13,23,24] The present study showed that treatment with Cl-Ad or MECA inhibited phosphorylation of Akt, decreased expression of Skp2, increased levels of $p27^{Kip1}$, and decreased expression of cyclin D1. These results are entirely consistent with our observations in SMCs[13] and support this mechanism of action for adenosine's antiproliferative effects in P-SMCs. The role of A_{2B} receptors in modulating these key signaling mechanisms to negatively influence cell proliferation is further supported by our observation that the effects of Cl-Ad and MECA on these signaling pathways were blocked by the A_{2B} receptor antagonist MRS and by silencing of A_{2B} receptors using siRNA.

Our results demonstrate that treatment with Cl-Ad reduces Skp2 expression and this is accompanied by a simultaneous increase in $p27^{Kip1}$ levels. Additionally, using pharmacological agonists and antagonists, we show that the modulatory effects of adenosine on Skp2 and $p27^{Kip1}$ are A_{2B}-receptor mediated. Skp2 is an F-box protein of SCFSkp2 ubiquitin ligase and therefore promotes polyubiquitination of and subsequent proteolysis of $p27^{Kip1}$.[23,24,30,31] Because $p27^{Kip1}$ binds to and inhibits the function of cyclin-Cdk complexes (such as cyclin D/Cdk4/6), an increase in $p27^{Kip1}$ levels would inhibit the function of cyclin D. Consistent with this mechanism, the present study supports the conclusion that via A_{2B} receptors adenosine inhibits P-SMC proliferation in part by downregulating Skp2 and upregulating $p27^{Kip1}$.

Our studies are consistent with the concept that the proximal signaling mechanism by which A_{2B} receptors inhibit

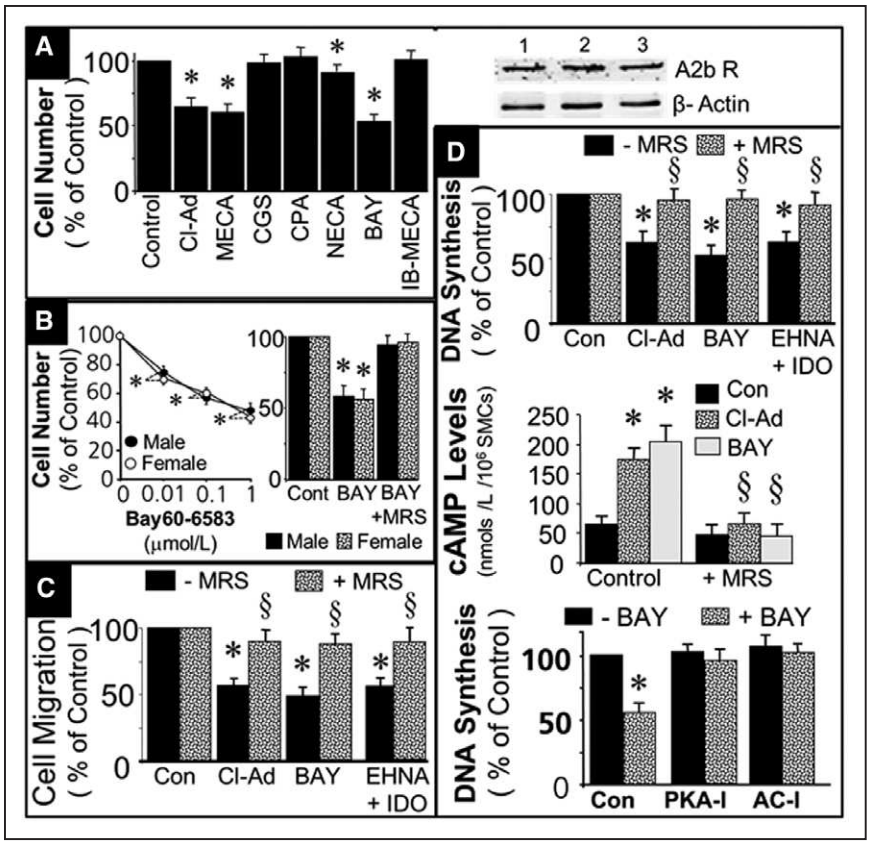

Figure 5. **A**, Depicts the expression of A_{2B} adenosine receptors and the inhibition of cell number by Cl-Ad, N-methylcarboxamidoadenosine (MECA), IB-MECA, N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), BAY, and CGS (100 nmol/L) in male progenitor smooth muscle cells (P-SMCs) cultures. **B**, Shows concentration-response relationships for the inhibition of cell number by BAY in male and female P-SMCs, and the reversal of their inhibitory effects by MRS (100 nmol/L). **C**, Summarizes the inhibitory effects of Cl-Ad, BAY, and EHNA (5 μmol/L) plus IDO (0.1 μmol/) on male P-SMC migration in the presence and absence of MRS. Top (**D**) depicts inhibitory effects of of Cl-Ad, BAY, and EHNA plus IDO on DNA synthesis in the presence and absence of MRS in male P-SMCs; middle (**D**) depicts the stimulatory effects of Cl-Ad and BAY on cAMP levels in male P-SMCs in the presence and absence of MRS; bottom (**D**) depicts DNA synthesis in male P-SMCs treated (+BAY) or not (–BAY) with BAY (0.5 μmol/L) in the presence or absence of a PKA (protein kinase A) inhibitor (myristoylated trifluoroacetate, frag 14-22; 10 μmol/L; PKA-I) or adenylyl cyclase inhibitor (2',5'-dideoxyadenosine; 1 μmol/L; AC-I) for 36 h. Both PKA-I and AC-I reversed the inhibitory effects of BAY on DNA synthesis in male P-SMCs. *P<0.05 vs no agonist; §Significant reversal of effects. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates.

proliferation involves the adenylyl cyclase/cAMP/PKA axis. A_{2B} receptors induce cAMP formation via activation of adenylyl cyclase.[13] Therefore, cAMP may be involved in mediating the effects of Cl-Ad on Akt and Skp2. Our findings that the inhibitory effects of Cl-Ad on P-SMC proliferation were significantly abrogated by inhibition of adenylyl cyclase and PKA are consistent with the hypothesis that A_{2B}-mediated cAMP production participates in the antimitogenic effects of Cl-Ad. We also observed that in P-SMCs with siRNA-silenced A_{2B} receptors, Cl-Ad-induced cAMP production was abrogated and the antimitogenic effects of Cl-Ad and MECA, but not 8-bromo-cAMP, were prevented. Taken together, these data suggest that the antiproliferative effects of Cl-Ad are mediated by cAMP produced via stimulation of A_{2B} receptors coupled to adenylyl cyclase, followed by cAMP-induced activation of PKA. This notion is supported by a previous report that cAMP inhibits neointima formation via PKA activation and by downregulating Skp2 and upregulating $p27^{Kip1}$ in rat aortic SMCs.[23,30–32]

Recent studies provide strong evidence for a major role of $p27^{Kip1}$ upregulation in mediating antimitogenic actions.[23,30] Decreased or defective expression of $p27^{Kip1}$ is linked to proliferative disorders including atherosclerosis, restenosis after balloon injury and cancer. In animal models, molecular approaches for targeted upregulation of $p27^{Kip1}$ prevent injury-induced intimal thickening.[31] Our finding that Cl-Ad induced $p27^{Kip1}$ expression in P-SMCs via A_{2B} receptors suggests that $p27^{Kip1}$ mediates in part the antimitogenic effects of Cl-Ad. Consistent with this notion, our experiments show that the stimulatory effects of Cl-Ad on $p27^{Kip1}$ expression and its inhibitory effects on P-SMC proliferation are blocked by A_{2B} receptor antagonism or knockdown of A_{2B} receptors.

Using the balloon vascular-injury model, we have previously shown that Cl-Ad inhibits neointimal formation.[13] Moreover, we demonstrated that treatment with Cl-Ad inhibits proliferating cells within the neointima. In the present study, Cl-Ad inhibited infiltration of c-Kit+ P-SMCs. Moreover, via A_{2B} receptors Cl-Ad inhibited P-SMC growth by downregulating Akt phosphorylation, Skp2 expression and cyclin D1 expression and upregulating $p27^{kip1}$ expression, a profile which was also observed by us in the neointimal tissue after injury in the same model.[13] Taken together, these findings suggest that Cl-Ad prevents intimal thickening in part by down-regulating the expression of Skp2 and upregulating $p27^{Kip1}$ levels. These findings are consistent with reports that injury-induced intimal thickening[16] and high-lipid-diet-induced atherosclerosis[33] are increased in mice lacking A_{2B} receptors, suggesting that the antivasoocclusive effects of adenosine are A_{2B}-receptor mediated.

Our findings that application of Cl-Ad peri-arterially inhibits injury-induced neointimal thickening has potential therapeutic significance. Restenosis is a major and frequent complication after balloon-angioplasty for the treatment of occlusive coronary artery disease. As increased presence of c-Kit+ cells[4,5] and abnormal growth of SMCs in neointima occurs mainly during the first 7 days post-angioplasty, and since peri-arterial application of Cl-Ad inhibits intimal thickening (likely via A_{2B} receptors), application of an A_{2B}-receptor agonist to the disease segment of the coronary artery at the time of angioplasty may prevent restenosis after balloon angioplasty in humans. Peri-arterial application may also resolve the limitations associated with the rapid clearance, short half-life, or adverse systemic effects of adenosine or its analogs.

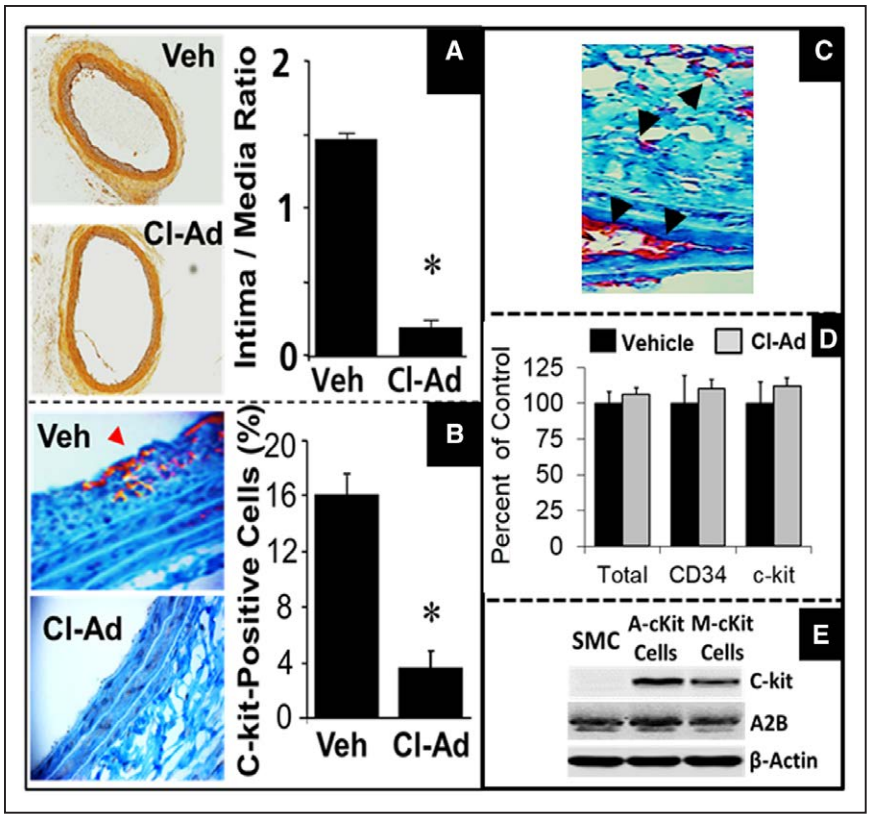

Figure 6. A, Shows the inhibitory effects of Cl-Ad (20 μmol/L in 25% pluronic gel) on intimal thickening after balloon injury. Image shows representative photomicrographs (10× magnification) of the cross-sections of rat carotid arteries 7 days after balloon injury in vehicle (Veh) vs Cl-Ad-treated rats. Bar graph compares the intima-to-media ratio after injury in rats receiving vehicle (n=7) vs Cl-Ad. Data are mean±SEM; * indicates $P<0.05$ vs vehicle. **B**, Depicts inhibitory effects of Cl-Ad on infiltration and presence of c-Kit positive cells within the intima 7 days after balloon injury (brownish-red staining using c-Kit antibodies). Image shows representative photomicrographs (40× magnification) of cross sections of carotid arteries stained for c-Kit. Bar graph compares the number of c-Kit positive cells in vehicle vs Cl-Ad treated groups. Data are mean±SEM; * indicates $P<0.05$ vs vehicle. **C**, Shows representative photomicrograph (100× magnification) depicting the presence of c-Kit positive cells within the vascular adventitia 7 days after balloon injury. In (**D**), the bar graph shows no difference in the number of marrow-derived CD34+ and c-Kit+ cells from rats treated with vehicle or Cl-Ad. **E**, Depicts Western blots demonstrating the presence of A_{2B} receptors on SMCs and c-Kit+ cells magnetically separated from enzymatically dispersed cells from rat aortas (A-cKit cells; n=7) and from bone marrow cells (M-cKit cells; n=7) using c-Kit antibodies.

Our findings demonstrate that peri-arterial application of Cl-Ad significantly decreases the number of c-Kit+ cells within the neointima; however, the source of these cells remains unclear. It is well established that marrow-derived c-Kit+ mononuclear cells contribute to neointimal thickening[2–5]; however, c-Kit+ cells have also been shown to be present within the vascular adventitia[2] and can contribute to the vascular remodeling process by migrating into the intima after injury.[2–4] The decrease in neointimal cells in response to Cl-Ad was not due to decreased production of marrow c-Kit+ or CD34 +cells, as Cl-Ad treatment did not lower their numbers. We found, however, that c-Kit+ cells were also present in the vascular adventitia. Hence, it is possible that both circulating and adventitial c-Kit+ cells can migrate to the site of injury and contribute to neointimal formation. This contention is supported by our finding that Cl-Ad inhibits P-SMC migration. It has been argued that the early accumulation of c-Kit+ cells in the luminal aspect of injured vessels is most likely derived from the circulation/marrow; however, concrete evidence remains elusive.[4,5]

The contribution of c-Kit+ cells in neointimal formation varies from 6% to 60%.[4,5] In the present study, the number of c-Kit+ cells, 7 days post-surgery, was ≈8%. As cells can lose expression of c-Kit+ with time, their true numbers within the neointima may be underestimated.[1,4] We have previously shown that Cl-Ad significantly inhibits the number of proliferating SMC-like cells in the neointima.[13] As similar to SMCs, adenosine inhibits growth of CD34+/c-Kit+ derived P-SMCs, it is likely that adenosine blocks proliferation of both SMCs and P-SMCs. However, due to lack of true/concrete specific markers, it is hard to differentiate between proliferating SMCs and P-SMCs.

Our finding show that A_{2B}-receptor activation inhibits in vitro growth of c-Kit-derived P-SMCs obtained from both females and males. This strongly suggests that A_{2B}-receptor agonists would counteract the vascular remodeling process associated with occlusive disorders in a sex-independent fashion. Nonetheless, as sex differences in adenosine responses are known to occur,[34] in vivo experiments are required to confirm our in vitro findings of sex independence.

Previous studies show that via A_{2B} receptors adenosine induces growth of CD34+-derived progenitor endothelial cells and drives them to initiate recovery at sites of endothelial injury[19,22] and that A_{2B} receptors are necessary for the early angiogenic process. These findings suggest that adenosine may protect against vascular remodeling by driving the relevant progenitors to repair the damaged endothelium (induce CD34+ progenitor endothelial cells) and by inhibiting P-SMCs that contribute to neointimal formation. Thus, it would be interesting to know if in injured/denuded vessels, application of Cl-Ad increases endothelial cell number and promotes endothelial recovery. Notably, A_{2B} receptors mediate anti-inflammatory actions and inhibit vascular adhesion of macrophages[21]; moreover, CD34+ hematopoietic cells can develop into both progenitor endothelial cells and SMCs.[2] As all of these cell types are represented within the developing neointima, we hypothesize that A_{2B} receptors prevent injury-induced neointimal formation by: (1) inhibiting proliferation and migration of vascular SMCs and CD34+/c-Kit+ derived P-SMCs; (2) inducing endothelial repair by activating growth, adhesion and angiogenesis of endothelial cells as well as CD34+ derived progenitor endothelial cells; and (3) inhibiting adhesion of pro-inflammatory monocytes/macrophages.

Based on our observations, the adenosine/A_{2B}-receptor system may protect against injury-induced vessel wall thickening; however, there are caveats. Overproduction of adenosine

and expression of A_{2B} receptors has been shown to induce deleterious actions and to be associated with the pathophysiology of lung fibrosis, chronic obstructive pulmonary disease, chronic renal disease and renal fibrosis, diabetes mellitus, cancer, wound healing, inflammatory conditions, autoimmune disease, cerebral ischemia and neurodegenerative disease, preeclampsia, and sickle cell disease.[35,36] As adenosine receptors, including the A_{2B}-receptor, can be desensitized by their ligand,[37] it is feasible that excessive adenosine production may have a negative impact on its receptor-mediated actions. Also, the time frame of A_{2B}-receptor activation may be critical, with short-term exposure providing beneficial vascular effects and long-term exposure promoting pro-fibrotic actions. From the therapeutic perspective, several adenosine receptor agonists and antagonists, as well as adenosine synthesis inhibitors, are under development for clinical use.[38,39] A_{2B} agonists are being developed to target cardiovascular disease[14]; whereas, A_{2B} antagonists and CD73 inhibitors are being developed to target angiogenesis in cancer/tumors,[40,41] renal fibrosis, pulmonary fibrosis associated with chronic obstructive pulmonary disease, pulmonary hypertension, and dermal fibrosis. As adenosine can have both beneficial and deleterious effects, the challenge going forward is to design drugs that maximize efficacy and minimize deleterious side effects.

In conclusion and as summarized in the graphical abstract, we provide strong evidence that: (1) adenosine inhibits neointimal formation and the infiltration of c-Kit+ cells; (2) adenosine inhibits proliferation and migration of human P-SMCs derived from CD34+/c-Kit+ mononuclear cells; (3) the inhibitory effects of adenosine on P-SMC proliferation are mediated via A_{2B}-receptor activation of adenylyl cyclase leading to the accumulation of cAMP; (4) adenosine inhibits P-SMC proliferation by activating PKA and blocking multiple pro-growth signaling pathways (Akt, Skp2, cyclin D1) and upregulating $p27^{kip1}$, a negative regulator of cell cycle progression; and (5) activation of A_{2B} receptors may prevent vascular remodeling associated with coronary artery disease, hypertension, atherosclerosis and restenosis.

Perspectives

We demonstrate that adenosine inhibits neointimal formation and P-SMC growth via A_{2B} receptors. Because infiltration and abnormal growth of P-SMCs importantly contributes to the vascular remodeling process associated with vaso-occlusive disorders, our results suggest that abnormal and decreased expression of A_{2B} receptors or decreased synthesis of adenosine may contribute to the abnormal growth of P-SMCs in various vaso-occlusive pathologies. Development of molecular approaches and pharmacological agents that activate and increase A_{2B} receptors may be of therapeutic importance in protecting against vascular remodeling associated with coronary artery disease, hypertension, atherosclerosis, and restenosis after balloon angioplasty.

Acknowledgments

Part of this work was presented, at the AHA Council for HBPR research meeting in Chicago 2009 (Dubey et al) and published as an abstract. All authors have substantially contributed to this work. R.K. Dubey and E.K. Jackson designed and supervised the study. R. Stiller and B. Imthurn were responsible for blood sample collection, I. Baruscotti for P-SMC isolation and characterization, Z. Mi for cAMP measurements, J. Fingerle for balloon injury animal experiments, M. Rosselli, R.K. Dubey, and D.G. Gillespie for conducting cell culture experiments and for immuno-staining, data collection, and analysis and equally contributed to the work; R.K. Dubey, E.K. Jackson, M. Rosselli, B. Leeners, and B. Imthurn wrote and edited the manuscript and figures.

The procurement of human blood for CD34+ mononuclear cell isolation was approved by the University Hospital Zurich ethics committee on human research. The animal experiments for balloon injury-induced neointimal thickening were performed in the laboratory or J. Fingerle and Hoffmann La Roche, Basel, and conformed to internationally accepted standards and approved by the appropriate institutional review body.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Endogenous and exogenous adenosine inhibit the proliferation and migration of human progenitor smooth muscle cells derived from CD34+/c-Kit+ circulating mononuclear cells.
- The inhibitory effect of adenosine on human progenitor smooth muscle cells is mediated via A_{2B}-receptor activation of adenylyl cyclase leading to the accumulation of cAMP and stimulation of PKA (protein kinase A).
- PKA inhibits human coronary progenitor smooth muscle cell proliferation by downregulating Akt-phosphorylation, Skp2, and cyclin D1 and by upregulating $p27^{kip1}$.
- Peri-arterial application of adenosine analogues in a slow release gel formulation inhibits the accumulation of c-Kit+ cells and attenuates vascular injury-induced neointimal hyperplasia.

What Is Relevant?

- A_{2B} receptor activation is a straightforward approach to inhibit both vascular and progenitor SMC proliferation and migration.
- Peri-arterial application of A_{2B}-receptor agonists may be of therapeutic importance in inhibiting neointimal hyperplasia while obviating unwanted systemic adverse effects.
- Adenosine and its A_{2B} specific analogs can abrogate neointimal progression by blocking growth of local and extravascular SMCs (adult and progenitor SMCs), which contribute to neointimal thickening.
- Other agents that modulate the actions of Akt, Skp2, p27Kip1, and cyclin D may have therapeutic efficacy in cardiovascular medicine.

Summary

The adenosine/A_{2B} receptor/cAMP/protein kinase A axis inhibits human coronary progenitor smooth muscle cell proliferation and migration by blocking multiple pro-growth signaling pathways (Akt-phosdphorylation, Skp2, cyclin D1) and upregulating $p27^{kip1}$, a negative regulator of cell cycle progression.